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GRANT NO: DAMD17-94-J-4201

TITLE: Effect of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

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REPORT DATE: July 27, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
July 27, 1995

3. REPORT TYPE AND DATES COVERED
Annual 1 Jul 94 - 30 Jun 95

4. TITLE AND SUBTITLE
Effect of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

5. FUNDING NUMBERS
DAMD17-94-J-4201

6. AUTHOR(S)
J. Mark Cline, D.V.M., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bowman Gray School of Medicine
Department of Comparative Medicine
Wake Forest University
Winston-Salem, NC 27157-1040

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The purpose of this study was to use histomorphometric and immunohistochemical techniques to study the incidence and characteristics of mammary gland hyperplasia, dysplasia and other possible indicators of breast cancer risk, in cynomolgus macaques given long-term hormonal treatments. Treatments evaluated to date include conjugated estrogens (CEE), medroxyprogesterone acetate (MPA), the combination of CEE and MPA, tamoxifen, estradiol (E2), and 17α-dihydroequilenin (DHEN). Pathologic evaluation, histomorphometric evaluations, and staining for estrogen receptor, progesterone receptor, and the proliferation marker Ki-67 MIB were done. Results are as follows: The addition of MPA to CEE therapy increases, rather than decreases, mammary gland proliferation. This finding is in contrast to the uterus, where MPA antagonizes the proliferation induced by CEE. Estrogen receptor expression in mammary gland epithelium is decreased by treatment with CEE, and further decreased by the addition of MPA. Progesterone receptor expression in mammary gland epithelium is increased by treatment with CEE, and decreased by the addition of MPA. Tamoxifen treatment does not induce mammary gland proliferation beyond that seen in controls; this is in contrast to a marked uterotrophic effect. DHEN does not induce mammary gland or endometrial proliferation, relative to controls and in contrast to E2.

14. SUBJECT TERMS
Breast Cancer; hormone replacement therapy; mammary gland; proliferation; steroid receptors; conjugated estrogens;medroxy-progesterone acetate; tamoxifen; phytoestrogens

15. NUMBER OF PAGES
54

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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J. Mark Clair 7/27/95

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INTRODUCTION

•Nature of the problem

Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease (Avila, 1990; Bush 1987; Henderson 1988; Hunt 1987; Petitti 1986; Stampfer 1985) and osteoporosis (Ettinger 1985; Kiel 1987; Weiss 1980; Ravnikar 1992; Colditz 1990). Unfortunately, the public health impact of these benefits to postmenopausal health has been small because of poor patient compliance. In the United States, 15% of naturally postmenopausal women 45 to 54 years of age use hormone replacement therapy (HRT), while the number decreases to 6% at ages 55 and older. A recent review of this problem indicates that **concern over the risk of breast cancer is the greatest single disincentive for the use of HRT** (Ravnikar 1992). Identification and quantification of this risk is compromised by a lack of basic knowledge of the hormonal regulation of the breast.

Studies of breast regulation, particularly in the postmenopause, are limited. Most studies have involved one of the following:
1. in vitro models which do not adequately mimic the hormonal milieu of the breast
2. in vivo studies in rodent models which are different in many ways from women
3. observational and epidemiologic studies of women which are confounded by lack of experimental rigidity.
4. studies in women using minimally invasive techniques such as fine-needle aspiration, which do not allow study of spatial/paracrine relationships within the breast.

Experimental studies using human tissues are subject to confounding errors: The apparently normal breast tissue in breasts operated for benign or malignant lesions may be subject to paracrine influence from the tumor, and the breasts of reduction mammoplasty operated women contain considerably more adipose tissue than the breasts from normal average women. Fine needle aspiration biopsies from healthy women also have limitations: Different epithelial areas of the mammary gland can not be safely distinguished in such samples. The ideal model would be based on surgical samples from healthy women, which is a practical impossibility.

Thus the problem lies in the lack of an adequate experimental system in which to test hypotheses relating to breast cancer risk. Our work is designed to use the cynomolgus macaque model to answer questions relating to breast regulation and breast cancer risk.

•Background of previous work

The concerns of women regarding breast cancer risk associated with hormonal therapies have some basis in the results of recent epidemiologic studies (Colditz 1992; Colditz 1993; Coope 1992; Pike 1993). Colditz et al found a significant association between current estrogen replacement therapy and increased breast cancer risk (Colditz 1992, 1993). The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestins on endometrial cell proliferation and cancer risk. The action of progestins on breast tissue is more controversial; the literature offers a number of conflicting results both *in vitro* (Mauvais-Jarvis 1986; Haslam 1988; Papa 1990; Moore 1991), and in vivo (Anderson 1989; de Lignieres 1992; Gompel 1993; Bergkvist 1989; Ohi 1992). The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e. estrogen + a progestin) is also appropriate for breast. However, a recent meta-analysis of studies including women treated with estrogen plus a progestin did not show a protective effect of the use of progestin (Colditz 1993). Recently, data from the Nurse's Health Study has shown a similar result (Colditz 1995).

The effects of various hormone therapies on atherosclerosis and osteoporosis in female monkeys have been studied for some time at our institution (Adams 1991, Clarkson 1989). The work described herein is an attempt to further utilize this model system for study of the breast. Several studies currently underway offer a unique opportunity for evaluation of the effects of estrogens, progestins, and androgens on mammary gland and endometrium *in vivo*, using animals given doses equivalent to those used in women. Therapies used include postmenopausal estrogen (Premarin) with and without a progestin (MPA); postmenopausal treatment with Premarin, MPA, the combination, or tamoxifen; premenopausal administration of Triphasil; premenopausal Triphasil followed by postmenopausal HRT; and postmenopausal nandrolone, an androgenic steroid. Studies are also in progress to assess the effects of dietary (soy) phytoestrogens. This collection of material represents a unique resource for the study of proliferative lesions induced in target tissues by hormonal replacement therapy.

Very few studies have dealt with the responses of macaque mammary glands to exogenous hormones (Speert 1948, Tavassoli 1988) Findings to date are that estrogens, progestins, and growth hormone can induce mammary gland proliferation, and that high doses of estrogens may induce neoplasms (Tavassoli 1988).

•Purpose of the present work

The specific aim of this work is to assess the effects of oral contraceptives and hormonal therapies on the incidence and severity of hyperplastic and dysplastic lesions in the mammary gland of macaques. The following hormone therapies are being evaluated.

<u>Treatments given to premenopausal animals</u>

Triphasil
Ethinyl estradiol (the more estrogenic component of Triphasil)
Levonorgestrel (the more progestinic component of Triphasil)

Treatments given to postmenopausal animals

Premarin
Premarin + medroxyprogesterone acetate
Medroxyprogesterone acetate
Nandrolone
Tamoxifen
17α- dihydroequilenin

•Methods of approach

Our basic approach is the use of intermediate markers of breast dysregulation in macaques (hyperplasia, dysplasia, epithelial proliferation measured by Ki-67 expression, and changes in estrogen and progesterone receptor expression) in order to identify which hormonal treatments might induce a greater risk of breast cancer in women. The associations between proliferative breast lesions and breast cancer risk are quite strong in women. Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation result in increased risk of transformation to the neoplastic phenotype (Cohen 1991, Butterworth 1992). Among women with benign breast lesions, ductal hyperplasia with atypia is a strong risk factor for the development of overt breast carcinoma (London 1992, Page 1988, DuPont 1985). Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well (Moolgavkar 1980).

Macaques are similar to women in many aspects of reproductive physiology and anatomy. Macaques have a distinct menarche and menopause, at about 3 and 20 years of age, respectively. They have a 28-day menstrual cycle, with a hormonal profile similar to that of women (Mahoney 1970). Their endometrial responses to endogenous and exogenous hormones parallel those of women (Kaiserman-Abramof 1989). Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar (Schultz 1937, MacPherson 1974, Benirschke 1978). Mammary neoplasms are uncommon in macaques (Benirschke 1978, Warner 1979). We believe that the female macaque model provides a unique alternative for measurement of responses of the breast to exogenous and endogenous hormones. This model also allows for extensive studies of treatment effects in normal breast which could not be done in women, for example serial biopsy studies or determinations of regional variations in breast regulatory processes.

BODY

•Experimental Methods

Study Design

Animals with a variety of hormonal manipulations are included in this work, as well as control monkeys from these studies, which allows concurrent study of the normal menstrual cycle. Studies from which tissues are being collected include the following:

Three studies dealing with postmenopausal hormone replacement therapy:

88-14 Estrogen replacement/secondary intervention trial
91-20 Estrogen replacement primary intervention trial
93-16 17α-dihydroequilenin study

One study using premenopausal (contraceptive) steroids and postmenopausal HRT:

91-12 Oral contraceptive atherosclerosis primary prevention trial

One study of contraceptive steroids alone:

91-24 Oral contraceptive study (Triphasil components trial)

One study of androgenic steroids used to prevent osteoporosis

92-04 Anabolic steroid study

A brief description of each study is given below.

Experiment 88-14

Estrogen replacement/secondary intervention trial

Study design

Progression phase - surgically postmenopausal, adult female monkeys were fed an atherogenic diet, to allow progression of atherosclerosis and osteoporosis for 22 months.

↓

Animals were then randomized into 3 groups:
- Ovariectomized control (n = 26)
- Premarin (n = 22)
- Premarin + Cycrin (n = 21)

↓

Treatment phase (24 months)
Diet changed to low fat and low cholesterol

↓

Euthanasia and necropsy
Termination date: July, 1993

↓

Assessment of:

Benefits: Atherosclerosis regression, arrest of osteoporosis progression.

Risks: Hyperplastic, dysplastic or neoplastic lesions in breast and endometrium. In particular, the relative effect of Premarin and Premarin + MPA.

Experiment 91-20: Primary intervention trial

Termination date: April 1995

Study design

Surgically postmenopausal, female cynomolgus monkeys

↓

Randomization to 5 groups:

- Ovariectomized control (n = 15)
- Premarin (n = 15)
- Cycrin (n = 15)
- Premarin + Cycrin (n = 15)
- Tamoxifen (n = 15)

↓

Treatment - fed moderately atherogenic diet, 35 months

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of Premarin alone and with Cycrin. Direct comparison of cardioprotective effect of Premarin and Tamoxifen.

Risks: Hyperplastic, dysplastic, and neoplastic lesions in breast and endometrium. This study is of particular interest because a) it allows comparison of the effects of Premarin and Premarin + MPA with MPA alone, and b) it provides an opportunity to examine the effect of Tamoxifen on normal mammary gland.

Experiment 93-16: 17α-dihydroequilenin study
Termination date: December 1993

Study design

Young, female rhesus monkeys

↓

Randomization to 3 groups:

Cycling control (n = 16)
Ovariectomized control (n = 17)
17α-dihydroequilenin (n = 17)

↓

Treatments were given for 21 weeks. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of 17α-dihydroequilenin.
Risks: Mammary hyperplasia, dysplasia, and neoplasia relative to either control group.

Experiment 91-12
Atherosclerosis/osteoporosis primary prevention trial
Termination date October 1993 (interim) and October 1995

<u>Study design (phase I)</u>
Premenopausal, female cynomolgus monkeys

↓

Randomization to 2 groups:
Control
Triphasil

↓

Treatment phase - Animals are fed a moderately atherogenic diet and treated for 24 months.

↓

Interim arterial biopsy

↓

Assessment of:
Benefits - cardioprotective effect of premenopausal estrogen use, particularly for stressed females. Increase in peak bone mass from premenopausal estrogen use.

↓

<u>Study design (phase II)</u>

Surgically postmenopausal monkeys

↓

Randomization of the two groups from part I (estrogen use or not) into control, Premarin and phytoestrogen groups, for treatment period of 36 months.

↓

Final necropsy

↓

Assessment of:
Benefits: Does premenopausal estrogen use add to the postmenopausal hormone replacement therapy effects on atherosclerosis and osteoporosis?
Risks: Hyperplasia and dysplasia of target tissues.
Possible protective effect of premenopausal contraceptive use on endometrium, and possible increased mammary tumor risk
What are the uterotrophic and mammotrophic effects of phytoestrogens?

Experiment 91-24
Atherosclerosis/contraceptive steroids primary prevention trial
Termination dates: June 1993 (interim sacrifice) and fall 1995

Study design

Premenopausal, female cynomolgus monkeys

↓

Randomization to 4 groups:

- Control (intact, normally cycling)
- Triphasil (n = 24)
- Cyclic ethinyl estradiol (n = 24)
- Cyclic levonorgestrel (n = 24)

↓

Treatment was given for 35 months. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of premenopausal estrogen use.

Risks: Mammary hyperplasia, dysplasia and neoplasia, and whether such effects relates to the ethinyl estradiol or levonorgestrel component of Triphasil.

Experiment no. 92-04
Osteoporosis primary prevention trial
Termination date: June 1994

Study design

Pre- and postmenopausal female cynomolgus monkeys

↓

Randomization:
- Cycling control (n = 15)
- Ovariectomized control (n = 15)
- Nandrolone in year 1 after ovariectomy (n = 15)
- Nandrolone in year 2 after ovariectomy (n = 15)

↓

Treatment was given for 24 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Benefits: Prevention/treatment of osteoporosis
Risks: Coronary artery atherosclerosis exacerbation. Effect of androgenic/anabolic steroids on mammary gland and endometrium.

Diets/Drug Dosing

The hormones were administered twice daily in the diet, with the exception of nandrolone. Most animals consume a moderately atherogenic diet (40% of calories from fat, 0.2 mg of cholesterol per Calorie). Monkeys are fed approximately 120 Calories per kg of body weight per day. Doses were as follows:

Drug		Abbreviation	Dose equivalent per woman per day
Conjugated equine estrogens		CEE	0.625 mg
Medroxyprogesterone acetate		MPA	2.5 mg
17β-Estradiol		E2	2 mg
Tamoxifen		TAM	20 mg
Ethinyl estradiol	Days 1-6	EE	0.03 mg
	Days 7-11		0.04 mg
	Days 12-21		0.03 mg
	Days 22-28		no drug
Levonorgestrel	Days 1-6	LN	0.05 mg
	Days 7-11		0.075 mg
	Days 12-21		0.125 mg
	Days 22-28		no drug
17α-Dihydroequilenin		DHEN	0.312 mg/kg
Soybean estrogens		SBE	99.7 mg
Nandrolone		-	No daily equivalent; animals were given injections of 25 mg nandrolone decanoate by intramuscular injection every 3 weeks.

Drug doses were computed as:

$$\frac{\text{human dose}}{\text{1800 Calories/woman/day}} = \text{dose per Calorie of diet}$$

Doses arrived at by this means were therefore consistently scaled, and adjusted for metabolic rate. They are similar to the dose calculated by scaling on the basis of body surface area (Mordenti 1986).

Tissue collection

Mammary glands were collected at the end of each study, when all monkeys are euthanized and necropsied. Tissues were fixed in 4% buffered paraformaldehyde at 4°C. The tissue was removed from paraformaldehyde after 24 hours, stored in 70% ethanol at 4°C, and later trimmed to 3 mm in thickness, embedded in paraffin using standard histologic procedures, and sectioned at 5 μm for immunostaining.

Histopathology

Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias.

Hyperplasia, atypia, cystic lesions, and secretory activity were noted. Lesions were independently graded as none, mild, moderate or severe. The criteria of the World Health Organization are used for tumor classification (WHO, 1982)

Morphometry
Mammary gland thickness, mammary lobular size, and area fraction of the mammary tissue occupied by glands are measured from histologic sections using video microscopy and a MacIntosh computer-based, public-domain image analysis program (NIH Image).

Stereology
In early studies prior to acquisition of the image analysis system, estimates of the relative proportions of tissue components in the mammary gland were made by point counting, after the method of Chalkley (Chalkely, 1945). These included percentage of gland occupied by epithelium, connective tissue and fat. Numbers of points intercepting each lobule were also recorded, as a relative indicator of lobular size.

Sex steroid receptors and proliferation marker staining methods.
Staining procedures were done on fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-peroxidase method (Wordinger 1987) modified for antigen retrieval from paraffin-embedded tissue. The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako laboratories (Dako Corporation, Carpinteria, CA, USA), and Immunotech laboratories (Immunotech, Marseille, France), respectively.

Assessment of proliferation (Ki67-MIB)
We use the KI-67 MIB-1 (MIB) monoclonal antibody (Immunotech, Marseille, France) that gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin embedded tissue sections (Cattoretti 1992). As for the receptor analysis, the MIB basic staining procedure is done by an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin embedded tissue. The murine monoclonal antibody Ki-67 reacts with a human DNA-binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell cycle analysis showed that the Ki-67 antigen is expressed in G1, S, G2 and mitosis (with maximum levels during G2 and M phases) but not in G0 and using this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible (Gerdes 1991).

Quantification of immunohistochemical staining
Immunostained cells were quantified by cell counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli, the terminal and major ducts were considered separately in order to assess regional differences. Labeled cell nuclei were identified as unlabeled (0), weakly (+), moderately (++), or intensely (+++) labeled. At least 100 cells per slide were counted at 3 different sites for each combination of animal, tissue site and

stain type. Major ducts and alveoli were easily identifiable, but clearly defined terminal ducts could not be identified in some cases.

Statistical methods

Statistical analysis is performed using the Mann-Whitney U-test with Bonferroni corrections for multiple comparisons, Kruskal Wallis test, Chi-square test, and Spearman's rank correlation test.

•Results obtained

A brief outline of accomplishments to date is followed by a presentation of experimental findings. Progress to date includes:

1. Collection of over 300 paired, frozen and fixed breast and endometrial samples, from macaques treated with conjugated estrogens with and without medroxyprogesterone acetate, tamoxifen, triphasic oral contraceptives, and dietary phytoestrogens.

2. Development of morphometric and cell counting methods for evaluation of breast and endometrium, including acquisition of a computerized video microscopy/image analysis system and development of standard measurement procedures.

3. Refinement and application of immunohistochemical methods for detection of the proliferation marker Ki-67, estrogen receptors and progesterone receptor; tissues from approximately 200 animals have been stained and evaluated to date.

4. Successful submission of a manuscript to the American Journal of Obstetrics and Gynecology detailing morphologic and immunohistochemical changes in the breast of surgically postmenopausal macaques given conjugated estrogens with or without the addition of MPA (from study 88-14). The manuscript is included as Appendix A.

5. Results in mammary gland have been compared to endometrial morphology and Ki-67, ER, and PR staining in endometrium, in the same animals used for the above manuscript (via a separate grant, received from the Office of Research on Women's Health). This parallel study has been recently presented at the Triangle Conference for Reproductive Biology (NC Biotechnology Center, Research Triangle Park, North Carolina), and has resulted in a Young Investigator Award being given to Dr. Cline for presentation of the findings at the North American Menopause Society meeting.

6. Study of the regional variation in breast regulation, by quadrant and distance from the nipple. Identification of regional variations provides us with an assessment of the degree of random or predictable intrinsic variation in within breast tissue, a parameter of considerable interest should we plan biopsy-based studies in the future.

7. Development of histopathologic criteria for retrospectively distinguishing the uteri of parous and nulliparous macaques. Since some animals in our studies were acquired as adults with an unknown reproductive history, and parity affects long-term breast regulation, this is an important source of variation in the breast which we needed to identify.

8. Other small ancillary projects were carried out which enhance the understanding of mammary data collected. These include development of vaginal cytology methods for prospective screening of live macaques for estrogenic effects on the reproductive tract, and development of a morphometric protocol to assess treatment-related changes in ovaries of oral contraceptive-treated macaques, in order to document treatment effects.

The following are the specific technical objectives proposed for 1994/1995 and 1995/1996, accompanied by a report of what has been accomplished.

Technical Objective	Work Accomplished
Year 1 (1994/1995)	
Processing, staining, and measurement from tissues collected in 1993; studies 88-14 (final sacrifice), 91-24 (interim sacrifice), 91-12 (interim sacrifice).	Completed.
Collection of tissues from studies 92-04 and 93-16.	Completed.
Year 2 (1995/1996)	
Collection of tissues from studies 91-20, 91-24, and 91-12 (final sacrifices).	Collections have been made from 91-20 and 91-24. Collections from 91-12 have been delayed by a change in the design of the parent study.
Processing, staining, and measurement of tissues collected.	This work is in progress as planned.
Presentation and publication of interim results from studies 91-24 and 91-12, final results from study 88-14.	Final results from study 88-14 (CEE+/-MPA) have been accepted for publication. Because of the high level of interest in results from studies 93-16 (17α-DHEN) and 91-20 (CEE, MPA, and tamoxifen), interim results from those two studies have been given precedence over those from studies 91-24 and 91-12. Interim data from the latter two will be presented later, along with their final results.

Specific Results Listed By Study

Substantial results are available from three studies, and are described below. Ancillary projects relevant to this work are described at the end of the results section.

I. Conjugated Estrogens with or without MPA (Study 88-14)
This is a long-term comparison of the effects of CEE and CEE+MPA in surgically postmenopausal macaques (the study design is shown on page 9).

Subjective Evaluation of Mammary Morphology

Mammary gland atrophy was seen in nearly all control animals. Animals given CEE alone had lobular atrophy or hyperplasia with equal frequency. Eighty-six percent of animals given CEE+ MPA had mammary hyperplasia, defined as greater mammary gland development than that seen in a normally cycling premenopausal macaque. Features similar to secretory differentiation were not related to treatment.

Morphometry and Stereology

Mammary gland thickness was significantly increased by treatment; the mean thickness (microns) $\pm$ SD was 264 $\pm$ 153 for controls, 396 $\pm$ 211 for animals given CEE, and 444 $\pm$ 249 for animals given CEE+MPA. Controls differed from both treated groups at $p < 0.05$, and from the CEE+MPA group at $p < 0.01$. The percentage of mammary gland occupied by glandular tissue was increased in both treated groups, most markedly in animals given CEE+MPA. Relative lobular size (expressed as points per lobule) was increased in animals given CEE, and more so in animals receiving CEE+MPA (Figure 1).

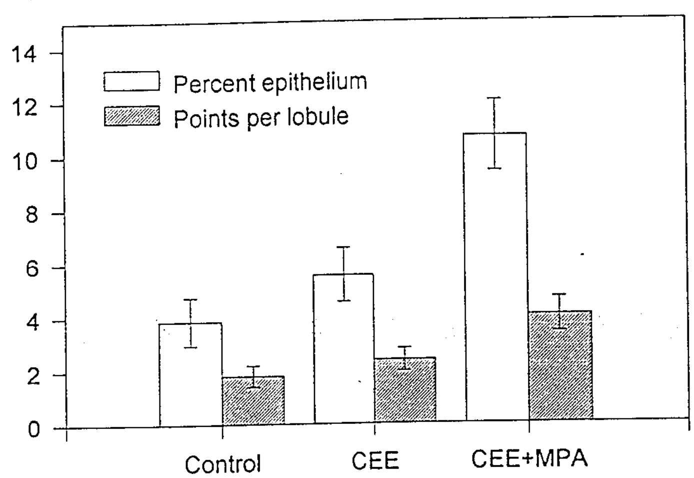


Figure 1. Changes in percent epithelium and lobular size (points/lobule) induced in macaque mammary gland by CEE or CEE+MPA.

Sex steroid receptors and proliferation

The percentage of estrogen receptor-positive cells was decreased in both treatment groups, most markedly in the CEE+MPA group (Figure 2). The percentage with positive staining for estrogen receptor and those intensely labeled (+++) was higher in the control and CEE groups than in the CEE+MPA group. Significant differences were found between the total number of receptor-positive cells in the CEE and CEE+MPA groups for alveoli, terminal ducts and major ducts. There were highly significant correlations between the percentages of estrogen receptor-positive cells of the alveoli and ducts $r_s = 0.69$, $p < 0.0001$, alveoli and terminal ducts $r_s = 0.70$, $p < 0.0001$, and between terminal ducts and ducts $r_s = 0.64$, $p < 0.0001$ (figure 3).

The percentage of progesterone receptor-positive cells was higher in the CEE group than in both the control and CEE+MPA groups in all three histologic sites (Figure 2). In all animals, there were highly significant correlations between the percentages of progesterone receptor-positive cells of the alveoli and major ducts $r_s = 0.76$, $p < 0.0001$, alveoli and terminal ducts $r_s = 0.64$, $p < 0.0001$ and between terminal ducts and major ducts $r_s = 0.64$, $p < 0.0001$ (Figure 3).

With regard to Ki-67 MIB staining, the treated groups in general had a larger proportion of proliferating cells than controls, with the highest proportion in the CEE+MPA group (Figure 2). Significantly higher values were seen in the CEE+MPA group relative to untreated controls in alveoli and major ducts, and there were also significantly higher values for the CEE group relative to controls for intensely labeled cells in the major ducts. There was a strongly significant correlation between percentages of positive cells in alveoli and terminal ducts ($r_s = 0.40$, $p = 0.0025$, Figure 3), and between alveoli and major ducts ($r_s = 0.32$, $p < 0.001$) but not between terminal ducts and major ducts.

Figure 2. Immunohistochemical staining for estrogen receptor, progesterone receptor, and the proliferation marker Ki-67 MIB in alveoli, terminal ducts, and large ducts.

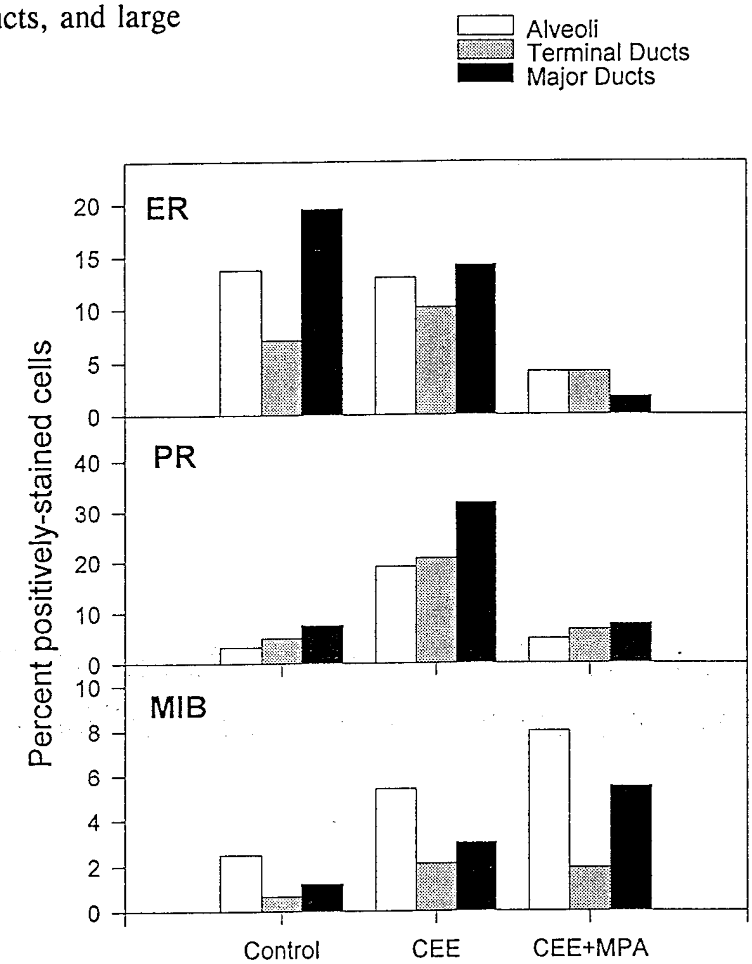
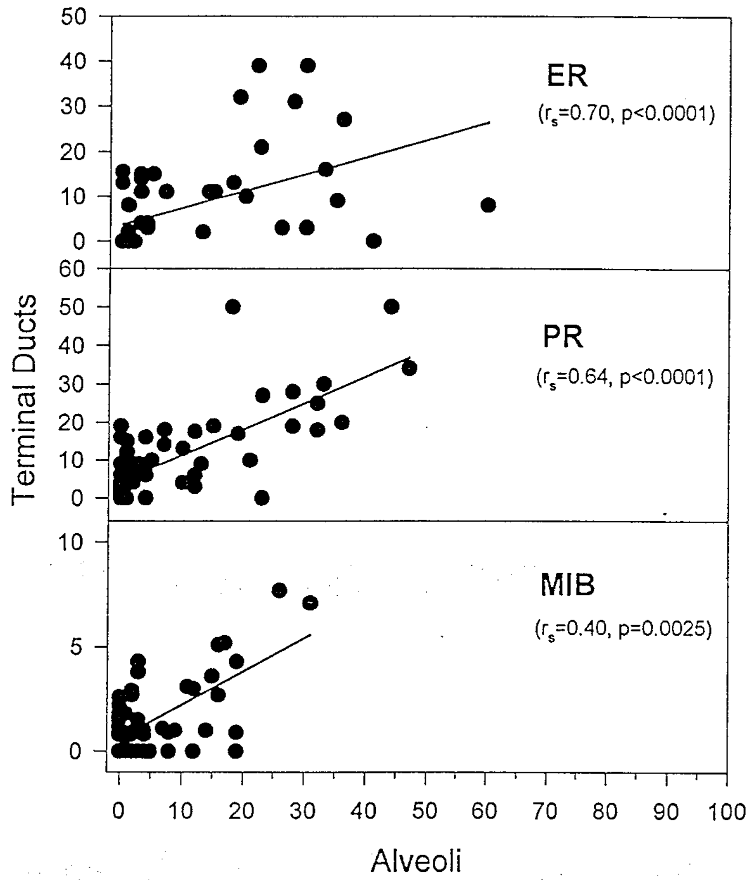


Figure 3. Correlations between different sites, with respect to immunohistochemical staining for estrogen receptor, progesterone receptor, and the proliferation marker Ki-67 MIB.



Regarding serum concentrations of hormones, the following correlations had an r_s of 0.25-0.5; all had a p value $\leq$ 0.05. *Higher serum concentrations of MPA* were positively correlated with MIB labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. Negative correlations were seen with ER and PR labeling. *Higher serum concentrations of estradiol* were positively correlated with MIB labeling, PR labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. A negative correlation was seen between serum estradiol and ER labeling.

When correlation testing was done for serum hormone concentrations within treatment groups, only MPA concentrations were positively correlated with any immunostaining parameter (alveolar cells with positive MIB staining, $r_s = 0.49$, $p = 0.035$, and strong alveolar staining, $r_s = 0.52$, $p = 0.024$).

When compared to our parallel studies of endometrium in the same animals, an interesting contrast is apparent. Endometrial thickness, epithelial MIB staining, and percentage of the endometrium occupied by glandular tissue were greatest in the group given CEE (figures 4 and 5). Combined therapy resulted in decreased endometrial thickness (2.04mm vs 2.6 for CEE alone), and less epithelial cell proliferation (0.8 vs 1.72%, nsd).

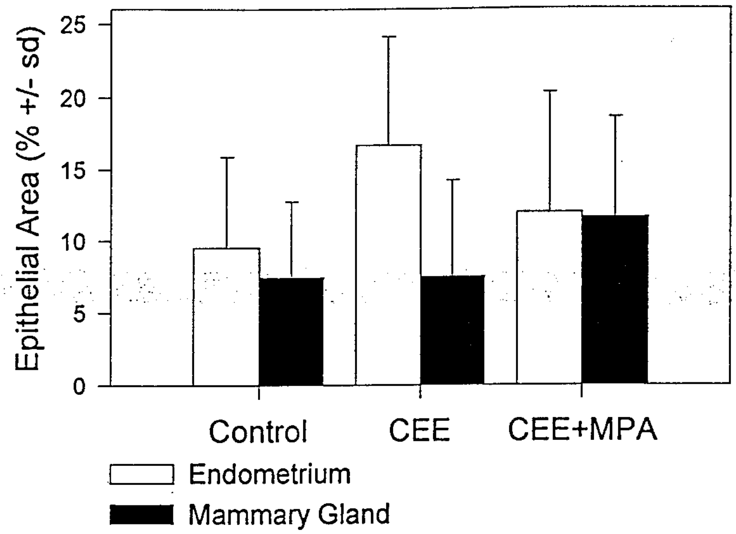


Figure 4. Morphometric comparison of mammary and endometrial proliferation induced by hormone replacement therapies.

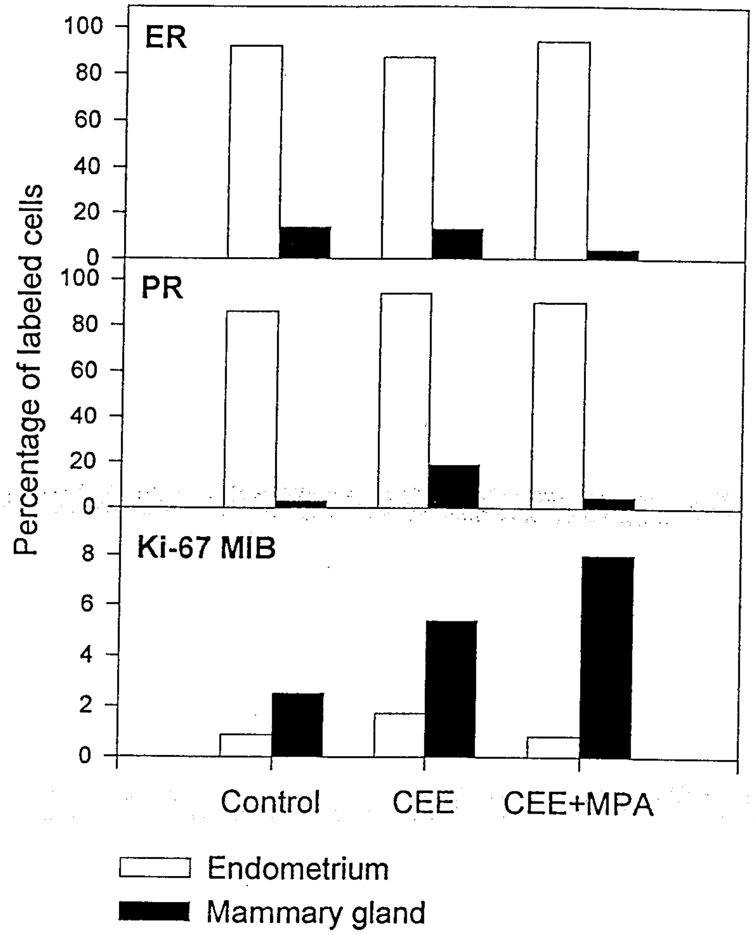


Figure 5. Comparison of sex steroid receptor staining and staining for the proliferation marker Ki-67 in mammary gland and endometrium.

These results show a proliferative response of the mammary gland epithelium to CEE+MPA in postmenopausal macaques, in the face of decreased ER and PR, and in contrast to the antagonistic effects of CEE and MPA on the endometrium. The findings reflect important regulatory differences between breast and endometrium, which are of particular relevance to the prediction of cancer risk posed by hormonal therapies.

The distribution of sex steroid receptors and proliferation (Ki-67 MIB expression) was explored in 10 sites from 12 animals in the above study. Quadrant of the breast and distance from the nipple did not have a significant effect on receptor expression or proliferation (figures 6 and 7).

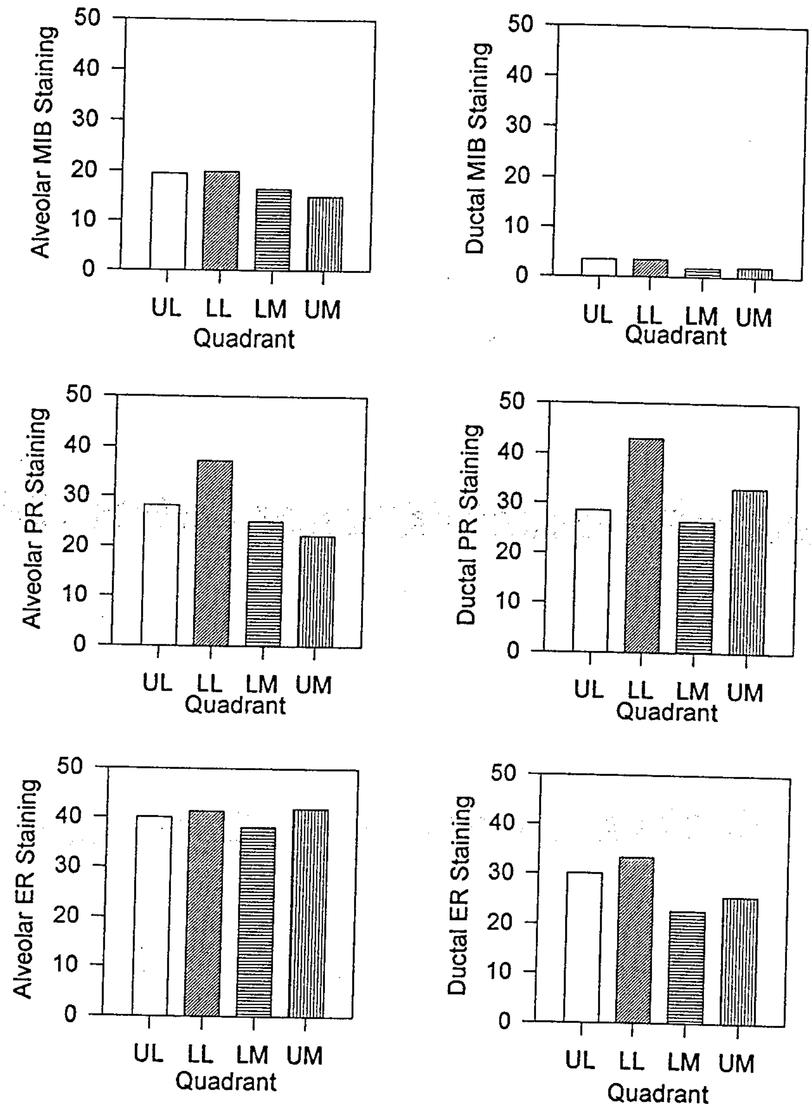


Figure 6. Regional distribution of mammary gland receptor expression and proliferation, by quadrant of the gland. Values are percentages of epithelial cell stained. UL = upper lateral quadrant; LL = lower lateral quadrant; LM = lower medial quadrant; UM = upper medial quadrant.

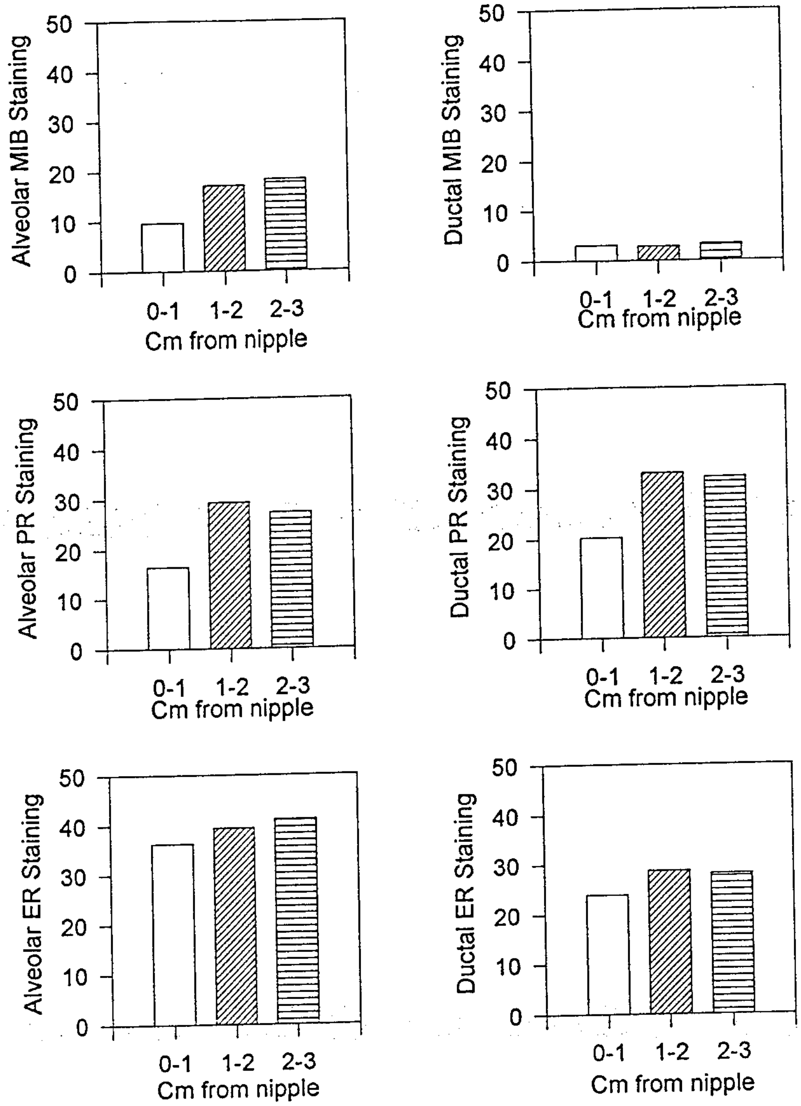


Figure 7. Regional distribution of mammary gland receptor expression and proliferation, by distance from the nipple.

There was a trend for increasing MIB labeling with increasing distance from the nipple, but statistical significance was not reached (p = 0.3561).

II. Conjugated estrogens, MPA and Tamoxifen (Study 91-20)
This is a recently terminated study of the comparative effects of CEE, MPA, CEE+MPA, and tamoxifen in surgically postmenopausal macaques (the study design is shown on page 10).

In this study, preliminary results in the groups given CEE, and CEE+MPA, are similar to those in the preceding experiment. Morphometric data on these groups, and the additional groups given MPA alone and tamoxifen, are shown in Figure 9. Again, it is apparent that CEE+MPA exert a greater mammotrophic effect than CEE alone, in contrast to the findings in the uterus. As might be expected, tamoxifen does not cause an increase in mammary gland thickness.

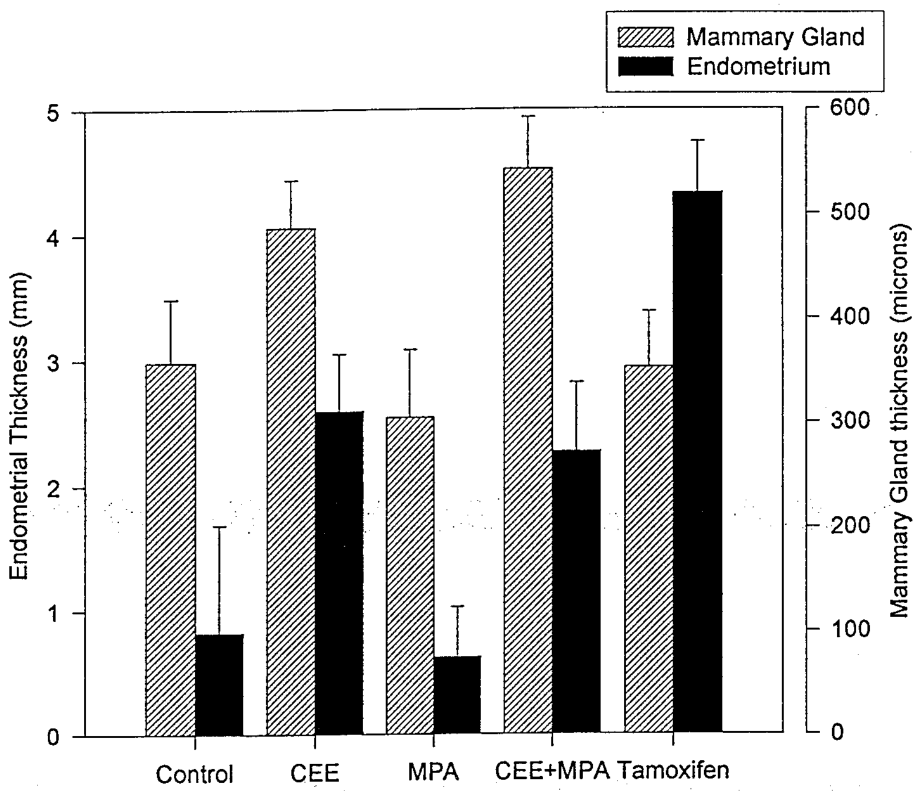


Figure 8. Comparison of mammary gland and endometrial thickness in animals given CEE, MPA, or Tamoxifen.

III. DHEN versus Conjugated Estrogens (Study 93-16)
This is a study of the relative effects of 17α-dihydroequilenin (DHEN) in pre- and post-menopausal macaques (the study design is shown on page 11).

In this study, DHEN did not exert mammotrophic or uterotrophic effects. Morphometric data illustrating this finding are shown in figure 9.

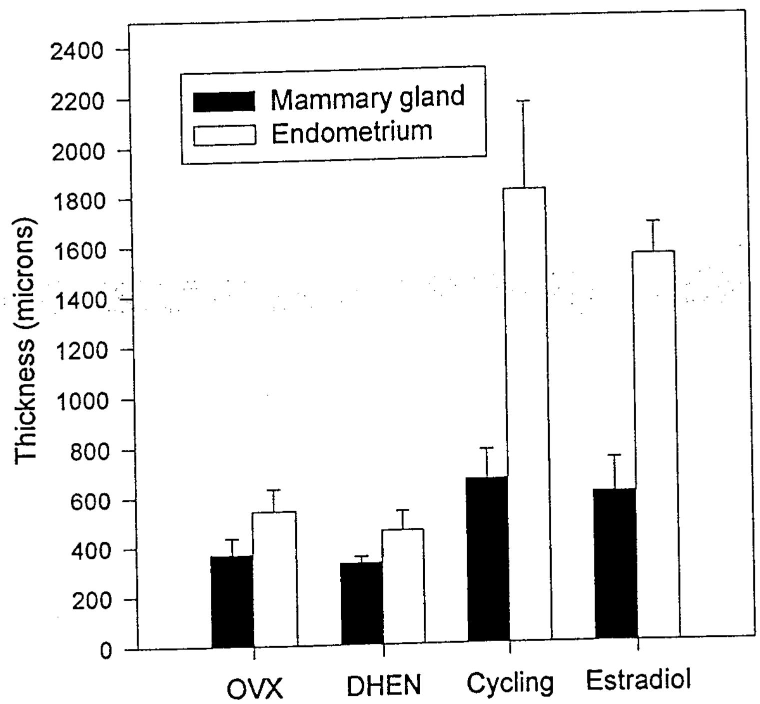


Figure 9. Mammary and endometrial thickness after administration of estradiol or 17-α dihydroequilenin (DHEN).

IV. Ancillary Projects

Identification of Previously Pregnant animals
The presence of perivascular extracellular mucinous matrix deposits was identified as an indicator of prior pregnancy. Using this criterion, 84/92 animals (91%) were correctly identified as to parity status. 2 out of 25 nulliparous animals (2% of the total) were incorrectly identified as parous; 6 out of 67 parous animals (7% of the total) were incorrectly identified as nulliparous.

Vaginal cytologic evaluation of hormone-treated animals
Vaginal cytologic examinations have been made on animals in studies 91-12 during the latter half of the experiment (see page 12 for the study design), and in study 91-20 (the study design is on page 10). Results to date indicate that CEE and tamoxifen exert an estrogenic effect on the vaginal epithelium, and that SBE does not (Figure 10).

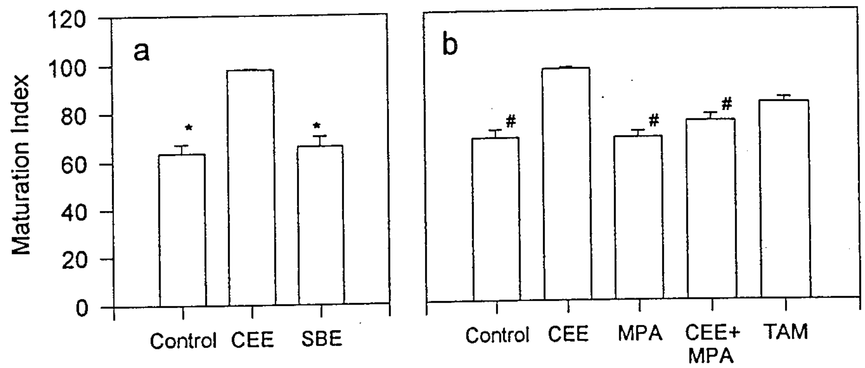


Figure 10. Vaginal maturation index for animals given hormonal or dietary treatments. Groups sharing symbols are not different at $p \leq 0.05$. a) - study 91-12; b) study 91-20.

•Discussion of results relative to goals of the research

The data presented herein clearly demonstrate that the macaque mammary gland can be used to provide a model of breast regulation in the post-menopausal period. This model is unique in that it provides an in vivo assessment of hormone effects on the primate breast, and can also be used to make comparisons of breast and endometrial effects in the same subject.

The most significant finding to date in this research is that the combination of CEE and MPA in this model does not result in suppression of the mammotrophic effects of CEE, but instead in a greater proliferative response than is seen with CEE alone. This finding addresses precisely the type of question the project is designed to target, providing a result which is of great relevance to public health, but which can only be explored with great difficulty in human subjects. The induction of proliferation in the breast by progestins is a subject of controversy. In the normal menstrual cycle of women, proliferation occurs primarily during the luteal phase of the cycle, indicating that breast does not respond to the same proliferative stimuli as the endometrium (Anderson 1989) It appears fairly certain, based on recent studies in women, that the addition of a progestin to estrogen replacement therapy does not protect the breast as it does the uterus (Colditz 1995). It remains to be seen whether or not there is an increased risk of breast cancer associated with combined HRT, as suggested by some investigators (Bergqvist 1989). Our data are in agreement with either possibility.

Another finding of importance is the contrast between mammary and endometrial effects of tamoxifen in macaques. Intuitively, this observation is in agreement with the antiproliferative and antitumor effects of tamoxifen in the human breast, and the increased risk of endometrial hyperplasia, polyps, and carcinomas caused by tamoxifen in women.

DHEN does not induce endometrial or mammary proliferation in this model. When this observation is grouped with previous finding at our institution that DHEN has beneficial effects on cardiovascular risk factors (Washburn 1993 and personal communications), it might be inferred that DHEN selectively confers the benefits of CEE therapy without the risks. Therefore, further exploration of the effects of DHEN and similar compounds is indicated. This drug serves as a paradigm for the exploration of novel therapies in the macaque model, and demonstrates the importance of evaluating multiple endpoints.

Among the ancillary projects, the most important work was the identification of histopathologic criteria which can be used to identify previously pregnant animals with a high degree of certainty. Given the importance of parity as a protective factor in women, it is imperative that as much information as possible is gathered about the reproductive histories of animals in our studies. We are happy to have identified what may be an important covariate in the analysis of data from this project. Vaginal cytologic measurements and ovarian histomorphometry will also enhance our understanding of the multisystemic effects of hormonal therapies. In particular, the vaginal cytologic measurements are a classic means of assessing the "estrogenicity" of a treatment.

CONCLUSIONS

General Summary:

Technical objectives outlined in the initial application have been met on schedule, with only minor changes.

The morphometric and immunohistologic methods proposed in the initial application have been applied successfully to a number of mammary gland samples from macaques.

Initial results have been accepted for publication in a peer-reviewed journal, and have resulted in an award from the North American Menopause Society to the principal investigator.

Specific Conclusions:

In surgically post-menopausal cynomolgus macaques,

The addition of MPA to CEE therapy increases, rather than decreases, mammary gland proliferation. This finding is in contrast to the uterus, where MPA antagonizes the proliferation induced by CEE.

Estrogen receptor expression in mammary gland epithelium is decreased by treatment with CEE, and further decreased by the addition of MPA.

Progesterone receptor expression in mammary gland epithelium is increased by treatment with CEE, and decreased by the addition of MPA.

Tamoxifen treatment does not induce mammary gland proliferation beyond that seen in controls; this is in contrast to a marked uterotrophic effect.

DHEN does not induce mammary gland or endometrial proliferation, relative to controls and in contrast to CEE.

Recommendations:

A great deal of important information can be gained within the scope of this project as initially written. However, after making the initial observations of hormone effects of these intermediate markers of cancer risk in breast, it will be vital to proceed on to more mechanistic studies of the role of growth factors and growth factor receptor expression in the proliferative response. The continuing controversy over breast cancer risk associated with hormonal therapies, particularly with regard to the role of progestins, indicates a lack of understanding of basic regulatory processes in the breast. The number of mediators potentially involved in breast regulation is large, including epidermal growth factor, insulin-like growth factor, relaxin, prolactin, tumor necrosis factor alpha, and others. The macaque model is ideally suited to the exploration of stromal-epithelial interactions in breast regulation, and this is and area we would like to pursue in future work. It appears unlikely that oncogene mutation or overexpression, or loss of a tumor suppressor gene will be found in this model system, given the lack of overt neoplasia; however, some exploration of these possibilities might be worthwhile also.

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Personnel Receiving Pay:

Name Social Security No.	Role	Percentage of Salary
J. Mark Cline	Principal Investigator	25%
Brian A. McCollough	Laboratory Technician II	50%
Shannon Schmotzer	Laboratory Technician II	50%

Graduate Degrees Received:
No graduate work was supported by this grant.

APPENDICES

Publications:
(see also Appendix A)

Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. J. Mark Cline, DVM, PhD[a]; Gunnar Soderqvist, MD[b]; Eva von Schoultz, MD, PhD[c]; Lambert Skoog, MD, PhD [d]; Bo von Schoultz, MD, PhD [b] Am J Obstet Gynecol 1995; In press.

Meeting Abstracts:
(see also Appendix B)

Addition of medroxyprogesterone acetate to conjugated equine estrogens in surgically postmenopausal macaques: Divergent effects on mammary and endometrial tissue. Cline, JM[1]; Soderqvist, G[2]; von Schoultz, E[3]; Skoog, L[4]; von Schoultz, B[2] Triangle Conference on Reproductive Biology, Research Triangle Park, NC, April 1995

Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Cline, JM[1]; Soderqvist, G[2]; Skoog, L[2]; von Schoultz, B[2] Accepted for presentation at the 6th Annual Meeting of the North American Menopause Society, San Francisco, CA, September 1995.

APPENDIX A

Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques

J. Mark Cline, DVM, PhD[a]; Gunnar Soderqvist, MD[b]; Eva von Schoultz, MD, PhD[c]; Lambert Skoog, MD, PhD [d]; Bo von Schoultz, MD, PhD [b]

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Supported by grant DAMD17-94-J-4201, US Army Medical Research Acquisition Activity, Fort Detrick, MD; by grants HL-38964 and HL-45666, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD; and by the Swedish Medical Research Council (project No. 5982); the Karolinska Institute Research Funds; and the Swedish Cancer Society.

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Condensation (25 word limit)

A comparison of the long-term effects of conjugated estrogens with and without medroxyprogesterone acetate in macaques, indicating greater breast proliferation in the combined treatment.

Abstract

Objectives: To define the proliferative response and receptor status in the mammary glands of surgically postmenopausal macaques given hormone replacement therapy equivalent for monkeys to that given women.

Study Design: Surgically postmenopausal adult female cynomolgus macaques (*Macaca fascicularis)* were given either no treatment (n = 26), conjugated equine estrogens (CEE, n = 22), or combined therapy with CEE and medroxyprogesterone acetate (CEE+MPA, n = 21). Drugs were administered in the diet, at doses equivalent on a caloric basis to 0.625 mg/woman/day for CEE and 2.5 mg/woman/day for MPA, for 30 months. Mammary gland proliferation was assessed subjectively and by morphometric and stereologic means. Estrogen receptor (ER) and progesterone receptor (PR) content and proliferation were studied using immunohistochemistry.

Results: In this model, combined therapy with CEE and MPA induced greater proliferation than CEE alone. The percentage of estrogen receptor-positive cells was decreased in the CEE+MPA group. The percentage of progesterone receptor-positive cells was increased by treatment with CEE alone.

Conclusion: These results indicate a proliferative response of mammary gland epithelium to CEE+MPA in postmenopausal macaques. The clinical implication of this finding may be a greater risk for development of breast neoplasms in women receiving combined hormone replacement therapy.

Key words: *Macaca fascicularis* • Hormone Replacement Therapy • Mammary gland • Steroid Receptors • Proliferation

Introduction

Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease and osteoporosis.[1] Unfortunately, the public health benefits of HRT have not been realized, largely because of the fear of cancer. A recent report indicates that among women in the US, concern over the risk of breast cancer is the greatest disincentive for the use of HRT.[2] This concern has some basis in the results of recent epidemiologic studies, which suggest an increased risk of breast cancer in long-term, current users of HRT.[1,3,4] The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestogens on endometrial cell proliferation and cancer risk. The great controversy concerns the action of progestogens on breast tissue, where the literature offers a number of conflicting results both *in vitro* [5-7], and *in vivo*.[8-11] The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e. estrogen + a progestogen) is also appropriate for breast. A recent meta-analysis of studies including women treated with estrogen plus a progestogen did not show a protective effect of progestogen use .[3]

Features of the Macaque Model

Rhesus and cynomolgus macaques are similar to women in many aspects of reproductive physiology and anatomy. Macaques have a distinct menarche and menopause, at about 3 and 20 years of age, respectively. They have a 28-day menstrual cycle, with a hormonal profile similar to that of women.[12] Their endometrial responses to endogenous and exogenous hormones parallel those of women.[13] Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar.[14] Human and macaque mammary glands display the same cytokeratin types.[15] Mammary neoplasms are uncommon in macaques.[16] This is the first large study of the mammary responses of macaques to long-term HRT.

Methods

Animals

The subjects of this study were 68 feral adult female cynomolgus monkeys (*Macaca fascicularis*) imported from Indonesia (Charles River Primates, Port Washington, NY). The animals were part of an atherosclerosis/osteoporosis prevention trial, the results of which will be published elsewhere. They ranged in age from 5 to 13 years as estimated from dentition and were not pregnant. Animals were housed in social groups of 4-8 monkeys each, in an AALAC-accredited facility; experimental protocols were approved by the institutional Animal Care and Use committee. Bilateral ovariectomies were done on all animals before the atherosclerosis induction period began.

Diets/Drug Dosing

The hormones were administered twice daily in a moderately atherogenic diet (40% of calories from fat, 0.2 mg of cholesterol per Calorie). Monkeys were fed approximately 120 Calories per kg of body weight per day. For 8 months of the 30-month treatment period, groups receiving CEE were given 7.2 μg of conjugated equine estrogens (Premarin®, Wyeth-Ayerst, Radnor, PA) per monkey per day. For the remaining 22 months, the dose was approximately 166 μg per monkey per day to be equivalent to women receiving 0.625 mg per day. Throughout the 30-month treatment phase, the group receiving CEE+MPA were given approximately 650 μg per monkey per day of medroxyprogesterone acetate (Cycrin®, Wyeth-Ayerst) to be equivalent to a woman's dose of 2.5 mg per day. Drug doses were computed as (human dose)/(1800 Calories/woman/day) = dose per Calorie of diet.

Serum Hormone Measurements

Prior to treatment, measurements were made of estradiol and progesterone to confirm completeness of ovariectomy; estradiol and medroxyprogesterone acetate were measured during the treatment phase. Samples were taken 4 hours after feeding/dosing. Medroxyprogesterone acetate was measured by radioimmunoassay. 17β-Estradiol was measured using a modification of a commercial kit (Diagnostic Products Corp.). All hormone measurements were carried out at the Comparative Endocrinology Laboratory of the Yerkes Regional Primate Center of Emory University (Atlanta, GA) by Dr. Mark Wilson.

Tissue collection

Mammary glands were collected at the end of the 30 month treatment phase, when all monkeys were euthanized and necropsied. Samples were taken in the sagittal plane through thenipple, and included a 2-3 cm segment of skin and gland. Tissues were fixed in 4% buffered paraformaldehyde for 24 hours and stored in 70% ethanol, at 4°C. Tissues were then trimmed to 3 mm in thickness, embedded in paraffin, and sectioned at 5 μm for immunostaining.

Histopathology

Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias. Hyperplasia, atypia, cystic lesions, and the presence of intralumenal protein or intraepithelial fat globules mimicking secretory activity were noted. Lesions were independently graded as none, mild, moderate or severe.

Morphometry and Stereology

Mammary gland thickness was measured as greatest thickness perpendicular to the skin from histologic sections. Measurements were made using an ocular micrometer at a magnification of 20x. An image analysis system (Bioquant, R and M Biometrics, Nashville, USA) was used to measure mean nuclear area and nuclear roundness factor in 10 randomly-selected cells each from alveoli, terminal ducts, and major ducts, at a magnification of 400x. Nuclear roundness factor is defined as $4\pi(\text{area})/\text{perimeter}^2$. Estimates of the relative proportions of tissue components in the mammary gland were made by point counting.[17] A 10 x 10 grid was superimposed on the section, and intercept points over features of interest were counted to determine the percentage of gland occupied by epithelium, connective tissue and fat. Numbers of points intercepting each lobule were also recorded, as a relative indicator of lobular size. For each section measured, 10 lobules were assessed, requiring an average of 4.6 microscopic fields at a magnification of 20x.

Sex steroid receptors and proliferation

Staining procedures were done on fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin-embedded tissue.[18] The estrogen receptor and progesterone receptor analyses were performed with reagents supplied by Dako laboratories (Dako Corporation, Carpinteria, CA, USA), and Immunotech laboratories (Immunotech, Marseille, France), respectively. To assess proliferation, we used the newly introduced KI-67 MIB-1 (MIB) monoclonal antibody (Immunotech, Marseille, France) that gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin embedded tissue sections.[18]

Quantification of immunohistochemical staining

Immunostained cells were quantified by cell counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli, the terminal and major ducts were considered separately in order to assess regional differences. Labeled cell nuclei were identified as unlabeled (0), weakly (+), moderately (++), or intensely (+++) labeled. At least 100 cells per slide were counted at 3 different sites for each combination of animal, tissue site and stain type. Terminal ducts could not be identified in some cases.

Statistical methods

Statistical analysis was performed using the Mann-Whitney U-test with Bonferroni corrections for multiple comparisons, Kruskal Wallis test, Chi-square test, and Spearman's rank correlation test.

Results

Hormone Measurements

Plasma estradiol concentrations (mean ± SEM) were 5.0 ± 0.7, 167.1 ± 9.9, 160.9 ± 13.9 pg/ml for controls, CEE and CEE + MPA groups respectively, $p < 0.0001$ between control and treatment groups. The corresponding medroxyprogesterone acetate concentrations were 35.9 ± 6.1, 24.7 ± 3.8 and 116.2 ± 5.2 pg/ml, $p < 0.0001$ between untreated and MPA-treated groups. MPA measurements for animals not given this drug were not significantly different from background.

Subjective Evaluation of Mammary Morphology

Mammary gland atrophy was seen in nearly all control animals. Animals given CEE alone had lobular atrophy or hyperplasia with equal frequency. Eighty-six percent of animals given CEE+ MPA had mammary hyperplasia, defined as greater mammary gland development than that seen in a normally cycling premenopausal macaque (see Table I). Features similar to secretory differentiation were not related to treatment.

Morphometry and Stereology

Mammary gland thickness was significantly affected by treatment; the mean thickness (microns) ± SD was 264 ± 153 for controls, 396 ± 211 for animals given CEE, and 444 ± 249 for animals given CEE+MPA. Controls differed from both treated groups at $p < 0.05$, and from the CEE+MPA group at $p < 0.01$. The percentage of mammary gland occupied by glandular tissue was increased in both treated groups, most markedly in animals given CEE+MPA. Relative lobular size (expressed as points per lobule) was increased in animals given CEE, and more so in animals receiving CEE+MPA (Figures 1 and 2).

Nuclear area was slightly increased in both hormonal treatment groups in the case of alveoli and terminal ducts. Nuclear roundness factor was slightly lower at all sites in the CEE+MPA group. Nuclear changes did not reach statistical significance.

Immunostaining for estrogen and progesterone receptors and proliferating cells

Table II illustrates the percentage of all receptor-positive cells for the different groups. The percentage of estrogen receptor-positive cells was decreased in both treatment groups, most markedly in the CEE+MPA group (Figure 3). The percentage with positive staining for estrogen receptor and those intensely labeled (+++) was higher in the control and CEE groups than in the CEE+MPA group. Significant differences were found between the total number of receptor-positive cells in the CEE and CEE+MPA groups for alveoli, terminal ducts and major ducts. There were highly significant correlations between the percentages of estrogen receptor-positive cells of the alveoli and ducts $r_s = 0.69$, $p < 0.0001$, alveoli and terminal ducts $r_s = 0.70$, $p < 0.0001$, and between terminal ducts and ducts $r_s = 0.64$, $p < 0.0001$.

The percentage of progesterone receptor-positive cells was higher in the CEE group than in both the control and CEE+MPA groups in all three histologic sites (Figure 3). In all animals, there were highly significant correlations between the percentages of progesterone receptor-positive cells of the alveoli and major ducts $r_s = 0.76$, $p < 0.0001$, alveoli and terminal ducts $r_s = 0.64$, $p < 0.0001$ and between terminal ducts and major ducts $r_s = 0.64$, $p < 0.0001$ (Figure 4).

Table III shows the proportion of MIB positive cells for the different histologic cell types and treatments. The treated groups in general had a larger proportion of proliferating cells than controls, with the highest proportion in the CEE+MPA group. Significantly higher values were seen in the CEE+MPA group relative to untreated controls in alveoli and major ducts, and there were also significantly higher values for the CEE group relative to controls for intensely labeled cells in the major ducts. There was a strongly significant correlation between percentages of positive cells in alveoli and terminal ducts ($r_s = 0.40$, $p = 0.0025$, Figure 4), and between alveoli and major ducts ($r_s = 0.32$, $p < 0.001$) but not between terminal ducts and major ducts.

Regarding serum concentrations of hormones, the following correlations had an r_s of 0.25-0.5; all had a p value ≤ 0.05. *Higher serum concentrations of MPA* were positively correlated with MIB labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. Negative correlations were seen with ER and PR labeling. *Higher serum concentrations of estradiol* were positively correlated with MIB labeling, PR labeling, lobular size, and percentage of the mammary gland section occupied by epithelial cells. A negative correlation was seen between serum estradiol and ER labeling.

When correlation testing was done for serum hormone concentrations within treatment groups, only MPA concentrations were positively correlated with any immunostaining parameter (alveolar cells with positive MIB staining, $r_s = 0.49$, $p = 0.035$, and strong alveolar staining, $r_s = 0.52$, $p = 0.024$).

Comment

Hormonal regulation of the normal breast, and hormonal risk factors for the development of breast cancer, remain a subject of controversy. In the normal menstrual cycle of women, proliferation occurs primarily during the luteal phase of the cycle, indicating that breast does not respond to the same proliferative stimuli as the endometrium.[19] Human and nonhuman primate mammary glands have many similarities, in terms of anatomy, hormonal regulation [14], and cytokeratin immunophenotype [15], that are not shared by the commonly used laboratory rodents. We believe that the macaque model offers a unique opportunity for study of mammary gland regulation, since it enables evaluation of the effects of long term HRT on various locations of the breast from healthy subjects.

Morphometric and stereologic evaluation of tissues in this study clearly indicate a mammotropic effect of CEE+MPA, which appears to exceed that of CEE alone. This study shows a down-regulation of both estrogen and progesterone receptors in breast epithelium during combined treatment similar to the endometrium. However, there was a significantly greater gland thickness and percentage of epithelial tissue on combined therapy than on CEE only. Also in contrast to the endometrium there is a clear trend of increased proliferative activity of the breast epithelium on combined therapy. This is in line with studies which suggest an increased breast cancer risk associated with combined estrogen-progestin therapy.[10] It is however important to note that there is no statistical difference between Ki-67 (MIB) labeling in ERT and HRT. The tendency for increased proliferation in the combined therapy group was accompanied by decreased proportions of progesterone receptor-positive cells. Previously our group [20] and others [4] have found a sustained progesterone receptor level under the influence of progesterone during the luteal phase of the menstrual cycle. These findings indicate that estrogen receptors in breast are down-regulated by progesterone, as in uterus [21], but that progesterone receptor positivity in breast does not change during the course of normal cycles. Progesterone is a well known stimulator of lobule-alveolar development. Apparently there are many differences between cyclic progesterone and continuous MPA. We have recently shown that progesterone increases the intra-tissue formation of estrone from estrone sulfate while norethisterone acetate in combined oral contraceptives does not, because of differences in sulfatase activity induced by these two compounds.[22]

The basis of risk associated with hormonal therapies may lie in regulation of cell proliferation. Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well.[4] The murine monoclonal antibody Ki-67 reacts with a human DNA-binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell cycle analysis showed that the Ki-67 antigen is expressed in G1, S, G2 and mitosis (with maximum levels during G2 and M phases) but not in G0 and using this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible.[23]

The proliferation of breast cells *in vitro* has mostly been found to be stimulated by estrogens and inhibited by progestogens. However, in these experiments cultured epithelial cells are deprived of their normal complement of blood vessels, adipose tissue, stroma and myoepithelial cells. These surrounding cells exert considerable paracrine and hormonal influence *in vivo*. Experiments with human tissue so far have been tritiated thymidine labeling and mitosis analyses on tissue sections from reduction mammoplasties or from "normal" breast tissue near a benign or malignant lesion. Most of these studies have demonstrated greater proliferation during the luteal phase.[4]

Clarke postulated that progestogens could activate the cell cycle for one turn, and that prolonged stimulation would turn it off.[24] Our study seems to contradict this hypothesis since two years of prolonged continuous combined therapy significantly enhances breast cell proliferation. There is one report of a direct stimulatory effect of 19-nor steroids on estrogen receptor-positive breast cancer cells via the ER.[25] The synthetic progestogen R 5020 stimulates insulin-mediated breast cancer cell proliferation by increasing insulin receptors and insulin receptor mRNA content; this may be another mechanism of action on normal breast epithelial cells.[7] Further studies from our group will evaluate this hypothesis and also sex steroid receptor variation, breast cell proliferation and growth factors of both cycling and oral contraceptive-treated healthy volunteers and cynomolgus monkeys.

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Acknowledgement:
We gratefully acknowledge the efforts of Torsten Hagerström, Berit Stäbi, Britt Kotander, Birgitta Bystrom, Audrey Kelleman, and Brian McCollough.

Legends

Figure 1. Typical estrogen receptor staining (a and b) and progesterone receptor staining (c and d) in control animals (a and c), and in animals from the CEE+MPA group (b and d). Positively-stained cells appear black. The loss of receptor staining is accompanied by an increase in the proportion of epithelial tissue.

Figure 2. Point counting measurements [16] of the percentage $\pm$ SEM of epithelium relative to stroma, and the number of points counted per lobule in the mammary gland of macaques. Both measures indicate glandular hyperplasia in the CEE+MPA group. For percent epithelium, the CEE+MPA group differs from the CEE group ($p < 0.05$) and from controls ($p < 0.0001$). For points per lobule, both CEE-treated and CEE+MPA-treated animals differed from controls ($p < 0.05$ and $p = 0.0007$, respectively), but the two did not differ from each other.

Figure 3. Immunostaining of mammary epithelial cells. MIB labeling is increased in both treatment groups, most notably in the CEE+MPA group. Estrogen receptor immunostaining is decreased in the CEE+MPA group. Progesterone receptor immunostaining is significantly increased only in the group given CEE alone. See Tables II and III.

Figure 4. Regression plots of correlations between immunostaining in alveoli and terminal ducts. Correlations are highly significant ($p < 0.0001$).

Table I
Numbers of animals with atrophic or hyperplastic mammary glands, by treatment. Numbers of animals with hyperplasia are significantly higher in the CEE+MPA group ($p = 0.0065$).

Treatment		Atrophy	Hyperplasia	Equivocal or not done
Control	n = 26	23 (92%)	0	2 (8%)
CEE	n = 22	11 (50%)	9 (41%)	2 (9%)
CEE+MPA	n = 21	2 (9%)	18 (86%)	1 (5%)

Table II
Mean percentage of receptor-positive breast epithelial cells from cynomolgus macaques. The number of evaluable specimens is indicated.

		ER		PR
		mean (range)		mean (range)
Alveoli				
A Control	n = 24	13.7 (0-63)	n = 25	3.2 (0-28)
B CEE	n = 22	12.9 (0-60)	n = 22	19 (0-47)
C CEE+MPA	n = 19	4.1 (0-28)	n = 19	4.8 (0-32)
Significant differences		BvsC, p = 0.014		AvsB, p = 0.0003 AvsC, p = 0.034 BvsC, p = 0.0006
Terminal ducts				
A Control	n = 18	7.1 (0-39)	n = 21	5.0 (0-27)
B CEE	n = 19	10.1 (0-39)	n = 19	20.7 (6-50)
C CEE+MPA	n = 18	4.1 (0-31)	n = 19	6.6 (0-25)
Significant differences		BvsC, p = 0.035		AvsB, p = 0.0003 BvsC, p = 0.0003
Major ducts				
A Control	n = 26	19.5 (0-76)	n = 26	7.5 (0-43)
B CEE	n = 22	14.1 (0-52)	n = 22	31.8 (0-56)
C CEE+MPA	n = 19	1.7 (0-18)	n = 19	7.7 (0-33)
Significant differences		BvsC, p = 0.003 AvsC, p = 0.007		AvsB, p = 0.0003 BvsC, p = 0.0003

Table III
Mean percentage of cells with MIB staining in breast epithelial cells of cynomolgus macaques. The number of evaluable specimens is indicated.

		MIB	
Staining intensity		All stained cells	+++ only
		mean (range)	mean (range)
Alveoli			
A Control	n = 25	2.5 (0-19)	0.08 (0-2)
B CEE	n = 22	5.4 (0-26)	0.14 (0-1)
C CEE+MPA	n = 19	8.0 (0-31)	0.84 (0-7)
Significant differences		AvsC, p = 0.016	AvsC, p = 0.009
Terminal ducts			
A Control	n = 20	0.6 (0-3)	0
B CEE	n = 22	2.1 (0-8)	0.04 (0-1)
C CEE+MPA	n = 19	1.9 (0-7)	0.13 (0-2)
Significant differences		-	-
Major ducts			
A Control	n = 26	1.2 (0-10)	0
B CEE	n = 22	3.0 (0-14)	0.32 (0-2)
C CEE+MPA	n = 19	5.5 (0-28)	0.84 (0-9)
Significant differences		AvsC, p = 0.017	AvsB, p = 0.015 AvsC, p = 0.046

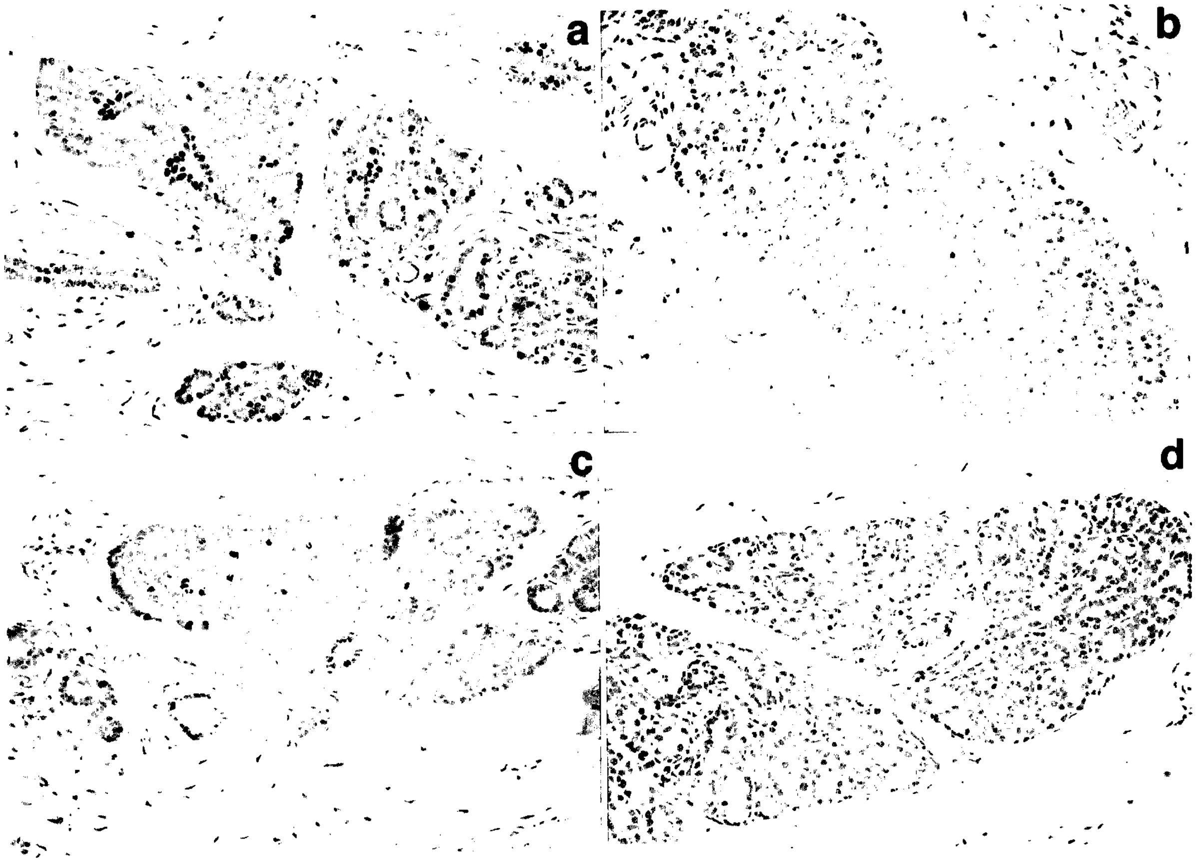


Figure 1

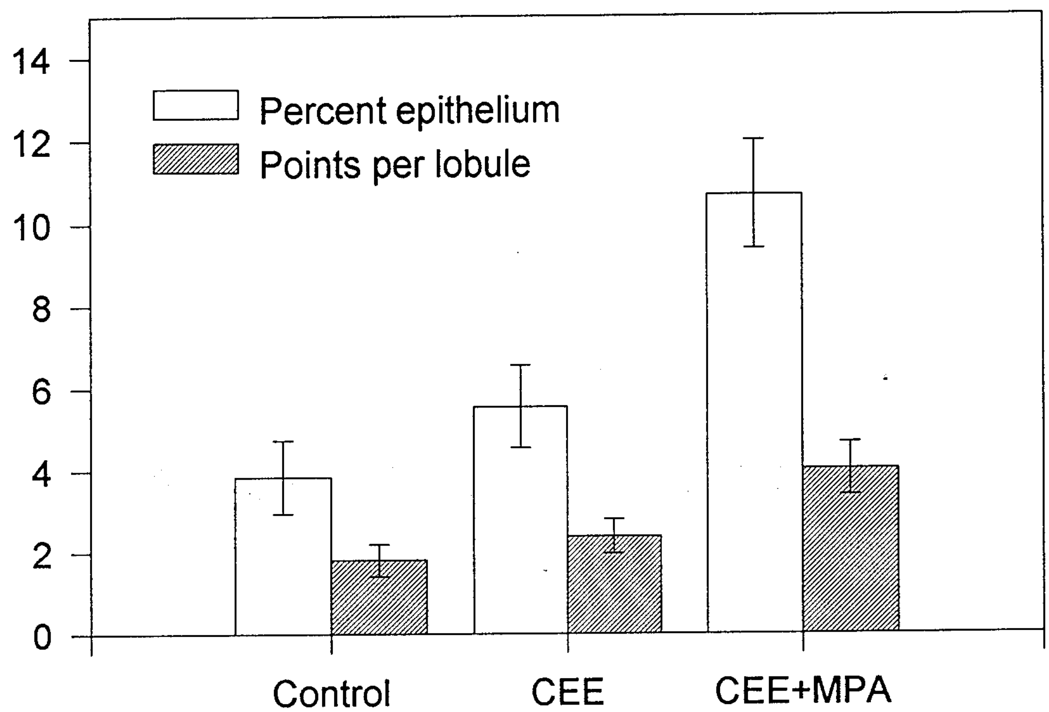

14
12
10
8
6
4
2
0
Percent epithelium
Points per lobule
Control
CEE
CEE+MPA

Figure 2

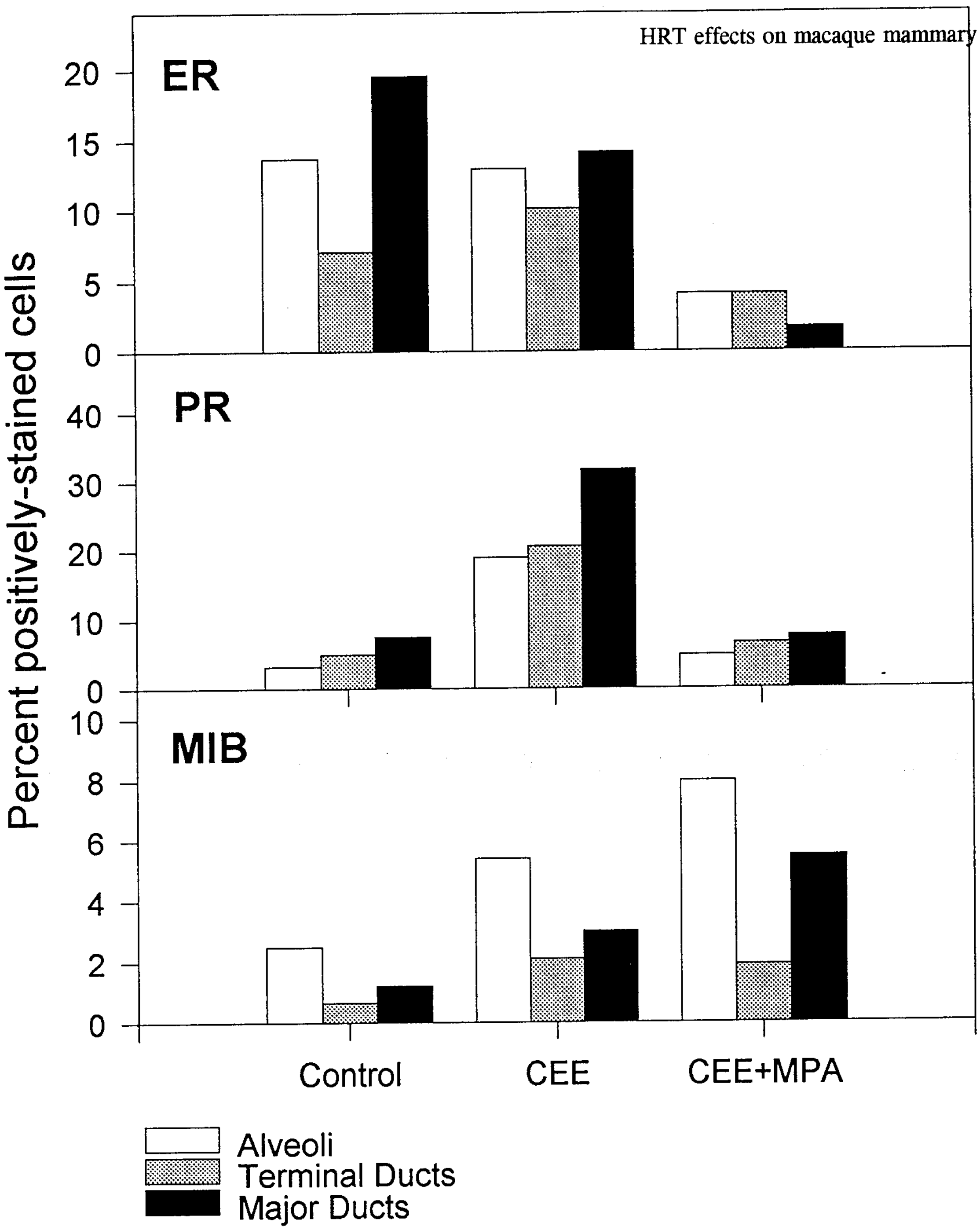
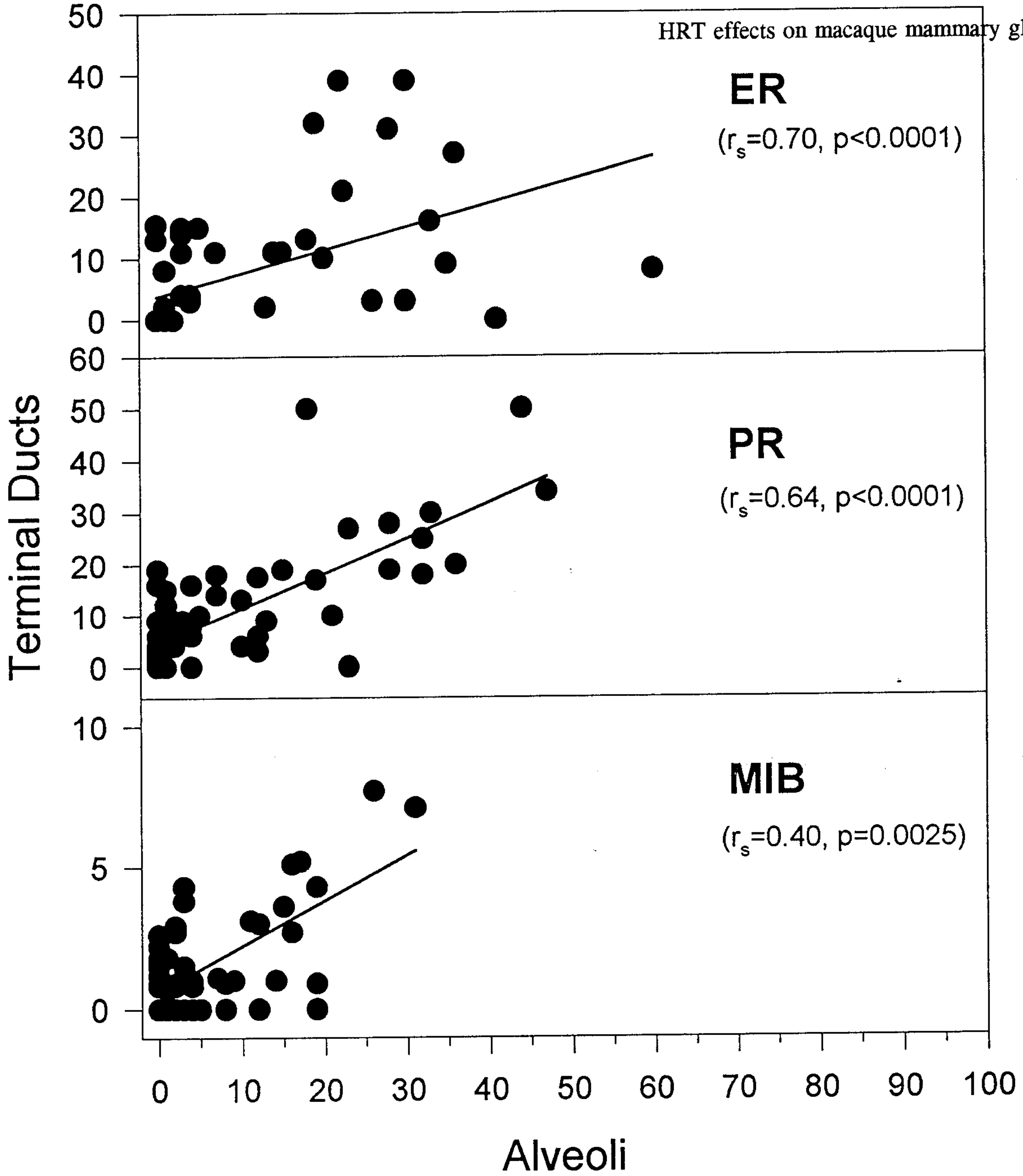

ER
PR
MIB
Percent positively-stained cells
20
15
10
5
0
40
30
20
10
0
10
8
6
4
2
0
Control
CEE
CEE+MPA
Alveoli
Terminal Ducts
Major Ducts

Figure 3



ER
(r_s=0.70, p<0.0001)
PR
(r_s=0.64, p<0.0001)
MIB
(r_s=0.40, p=0.0025)
Terminal Ducts
Alveoli
50
40
30
20
10
0
60
50
40
30
20
10
0
10
5
0
0
10
20
30
40
50
60
70
80
90
100

Figure 4

Appendix B: Presented at the Triangle Conference on Reproductive Biology, Research Triangle Park, NC, April 1995

ADDITION OF MEDROXYPROGESTERONE ACETATE TO CONJUGATED EQUINE ESTROGENS IN SURGICALLY POSTMENOPAUSAL MACAQUES: DIVERGENT EFFECTS ON MAMMARY AND ENDOMETRIAL TISSUE

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Surgically postmenopausal adult female macaques (*Macaca fasciculata*) were given oral conjugated equine estrogens (CEE, n = 22), combined therapy with CEE and medroxyprogesterone acetate (CEE+MPA, n = 21) or no treatment (n = 26), for 30 months. Doses were equivalent on a caloric basis to 0.625 mg/woman/day for CEE and 2.5 mg/woman/day for MPA. Mammary gland and endometrial proliferation were assessed by morphologic and immunohistochemical means at the end of the study. Tissues were stained for estrogen receptor (ER), progesterone receptor (PR), and the proliferation marker Ki-67 MIB (MIB). Results are as follows:

In mammary gland - Combined therapy with CEE+MPA induced greater proliferation than CEE alone, as indicated by point counting estimates of lobular size and glandular area fraction and proportions of cells with MIB staining (2-8% for CEE+MPA, <1% for others; p=<0.05 versus controls). The percentage of ER-positive cells was decreased in the CEE+MPA group (2-4% vs. 7-20% for other groups, p<0.03). The percentage of PR-positive cells was increased by treatment with CEE alone (20-30%, p=0.0003), compared to controls and CEE+MPA (3-7%).

In the uterus - Endometrial thickness, epithelial MIB staining, and percentage of the endometrium occupied by glandular tissue were greatest in the group given CEE. MIB staining was minimal in all groups (1-3% of epithelial cells). Combined therapy resulted in decreased endometrial thickness (2.04mm vs 2.6 for CEE alone), and less epithelial cell proliferation (0.8 vs 1.72%, nsd), but increased superficial and deep stromal cell proliferation (p>0.05). Treatment-related changes in ER and PR in endometrial epithelium were not significant; >85% of cells stained positively in each group. Significant increases in stromal PR were seen in both treatment groups, most markedly in the superficial stroma of animals given CEE+MPA (p=0.0002 vs controls, 0.05 vs. CEE alone). In the myometrium, ER positivity was around 70%. PR staining was seen in 30% of myocytes in controls and about 85% in either treatment group, and proliferation was absent.

These results show a proliferative response of the mammary gland epithelium to CEE+MPA in postmenopausal macaques, in the face of decreased ER and PR, and in contrast to the antagonistic effects of CEE and MPA on the endometrial epithelium. CEE+MPA additionally caused an increase in endometrial stromal PR and MIB staining, particularly in superficial stromal cells.

Supported by grants DAMD17-94-J-4201, USAMRAA; by HL-38964 and HL-45666, NHLBI, NIH; the Swedish Medical Research Council (project No. 5982); Karolinska Institute Research Funds; and the Swedish Cancer Society.

Appendix B: Abstract accepted for presentation at the 6th Annual Meeting of the North American Menopause Society. This abstract resulted in a Young Investigator Award.

DIVERGENT EFFECTS OF HORMONE REPLACEMENT ON MAMMARY AND ENDOMETRIAL TISSUES OF MACAQUES

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The purpose of this study was to evaluate cancer risk-related markers in a primate model of postmenopausal hormone replacement therapy. Surgically postmenopausal adult female macaques (*Macaca fascicularis*) were given oral conjugated equine estrogens (CEE, n = 22), CEE and medroxyprogesterone acetate (CEE+MPA, n = 21) or no treatment (n = 26), for 30 months. Doses were equivalent to 0.625 mg/woman/day for CEE and 2.5 mg/woman/day for MPA. Mammary and endometrial changes were assessed by morphologic and immunohistochemical means. Tissues were stained for estrogen receptor (ER), progesterone receptor (PR), and the proliferation marker Ki-67 MIB (MIB). **Results:** *In mammary gland* - CEE+MPA induced greater proliferation than CEE, by point counting estimates of lobular size, % epithelium and % of MIB-stained cells (2-8% for CEE+MPA, <1% for others; p=<0.05 vs. controls). ER+ cells declined in the CEE+MPA group (2-4% vs. 7-20% for others, p<0.03). The % of PR+ cells increased with CEE alone (20-30%, p=0.0003), compared to controls and CEE+MPA (3-7%). *In uterus* - Endometrial thickness, MIB staining, and percent glands were greatest in the CEE group. CEE+MPA decreased endometrial thickness (2.04mm vs. 2.6 for CEE alone), and decreased epithelial MIB staining (0.8 vs 1.72%, nsd). **Conclusion:** There is a proliferative response of mammary gland epithelium to CEE+MPA in postmenopausal macaques, in the face of decreased ER and PR, and in contrast to the antagonism of CEE and MPA in endometrium.